



Non-canonical activation of inflammatory caspases by cytosolic LPS in innate immunity

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Lipopolysaccharide (LPS) is the major component of Gram-negative bacteria cell wall. In innate immunity, extracellular LPS is recognized by Toll-like receptor 4 to stimulate cytokine transcription. Recent studies suggest a 'non-canonical inflammasome' that senses cytoplasmic LPS and activates caspase-11 in mouse macrophages. Unexpectedly, biochemical studies reveal that caspase-11 and its human orthologs caspase-4/caspase-5 are LPS receptors themselves. Direct LPS binding induces caspase-4/caspase-5/caspase-11 oligomerization and activation, triggering cell pyroptosis and anti-bacterial defenses. Caspase-4/caspase-5/caspase-11 recognition of intracellular LPS requires bacterial escape from the vacuole; this process is promoted by interferon-inducible GTPases-mediated lysis of the bacteria-containing vacuole. Non-canonical activation of these inflammatory caspases by LPS not only represents a new paradigm in innate immunity but also critically determines LPS-induced septic shock in mice.

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Introduction

Mammalian innate immunity relies on a family of pattern recognition receptors (PRRs) to detect conserved microbial molecules termed pathogen-associated molecular patterns (PAMPs) [1,2]. PRRs engagements by corresponding PAMPs activate inflammatory pathways such as the nuclear factor- κ B (NF- κ B) and interferon regulatory factor (IRF) signaling for cytokine transcription and clearance of the infections. The PRR family includes Toll-like receptor (TLR), C-type lectin receptor (CLR), RIG-I-like receptor (RLR), AIM2-like receptor (ALR) and NLR containing a Nucleotide-binding domain

(NBD) and a Leuclidean-rich Repeat (LRR) domain. TLR and CLR are localized on the plasma or endosomal membrane while RLR, ALR and NLR are cytoplasmic [3,4]. These enable the host to monitor both extracellular and intracellular infections. The best studied PRR is TLR4 that complexes with MD2 to recognize LPS [4], the major component of Gram-negative bacteria cell wall. Identification of TLR4 as the LPS receptor is paradigm-shifting in innate immunity [5].

Several NLRs and ALR, in response to certain PAMPs, form a canonical inflammasome complex for activating caspase-1 [6,7]. For example, the NAIP subfamily of NLRs, upon recognition of bacterial flagellin or type III secretion apparatus, undergoes hetero-oligomerization with an adaptor NLR (NLRC4) for inflammasome assembly [8–10]. Activated caspase-1 then processes interleukin (IL)-1 β and IL-18 for their maturation and at the same time induces pyroptosis, a lytic form of inflammatory cell death. Caspase-1 belongs to the inflammatory caspase group that additionally includes murine caspase-11, human caspase-4 and caspase-5, and also caspase-12. These caspases structurally resemble apoptotic initiator caspases, all bearing an amino-terminal caspase-activation and recruitment domain (CARD). A recent study suggests that caspase-11 can also induce pyroptosis in response to bacterial infections in a caspase-1 independent manner [11]. Analogous to caspase-1 activation by canonical inflammasome scaffolds, a 'non-canonical inflammasome' for caspase-11 activation was proposed. Here we review research progresses on the 'non-canonical inflammasome' in the past two years.

LPS activation of a caspase-11 'non-canonical inflammasome'

In studying toxin stimulation of inflammasome responses, Kayagaki *et al.* observed that cholera toxin B (CTB) could induce NLRP3/ASC-dependent IL-1 β maturation in LPS-primed mouse macrophages [11]. Surprisingly, this activation was absent in macrophages derived from strain 129 mice. The defect was attributed to the polymorphism in 129 mice-derived *caspase-11* that encodes a truncated and nonfunctional caspase-11 protein. This led to the discovery of caspase-11 function in sensing CTB as well as infections with extracellular bacteria such as *Escherichia coli*, *Citrobacter rodentium* and *Vibrio cholera*. A series of subsequent studies have now established that the caspase-11 'non-canonical inflammasome' generally responds to Gram-negative bacterial infections including

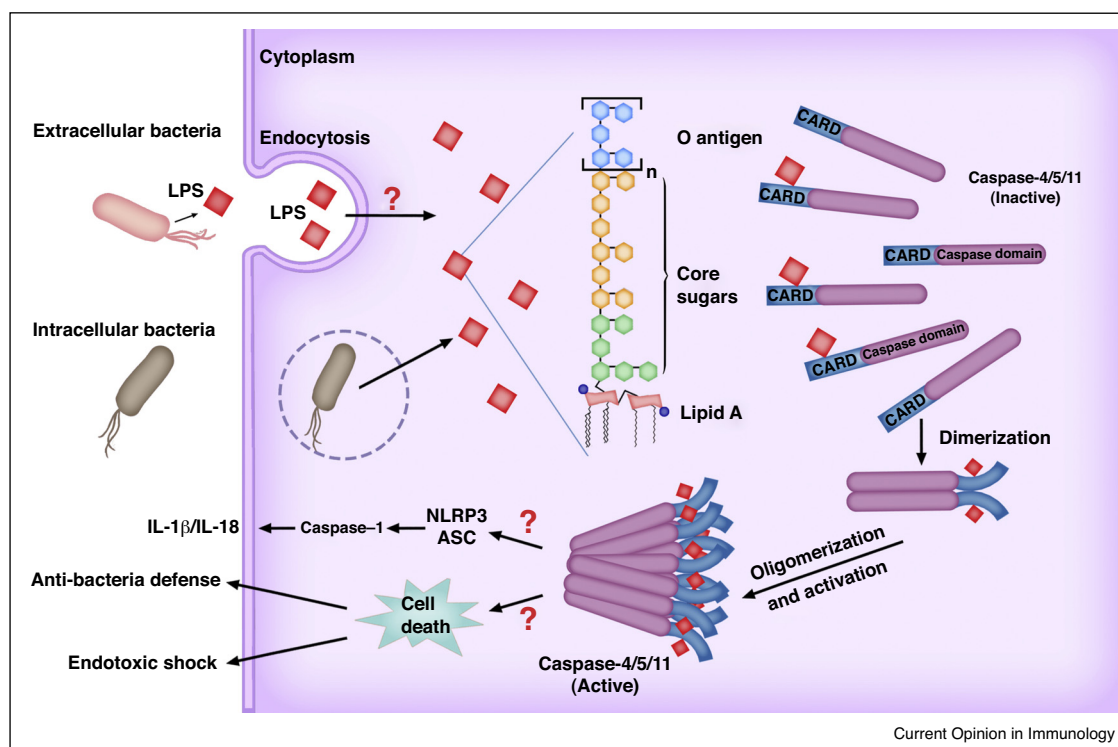
Legionella pneumophila, *Salmonella typhimurium*, enterohemorrhagic *E. coli* and *Burkholderia* spp. [12–14,15*,16].

Activation of caspase-11 signaling required 15–20 h of infection with extracellular bacteria but only 2 h for intracellular *L. pneumophila* [11,14,16]. Aachoui *et al.* further observed that *B. pseudomallei* and its low-virulence surrogate *B. thailandensis* that naturally escape from the vacuole induced rapid caspase-11-dependent pyroptosis [15*]. Caspase-11 activation by vacuole-living *L. pneumophila* and *S. typhimurium* was promoted by mutations in *sdhA* and *sifA*, respectively, both of which caused rupture of the vacuole and release of the bacteria. These observations support the notion that the caspase-11 axis detects cytosolic bacteria but not those residing in an intact vacuole [15*]. Two independent new studies have now identified LPS being the responsible bacterial agonist [17**,18**]. Kayagaki *et al.* followed the activity of LPS plus CTB and found that only LPS O111:B4 could work together with CTB in triggering caspase-11 activation. This was due to the unique O-polysaccharide in LPS O111:B4 that could

bind to CTB. Thus, CTB only played a carrier role for cytosolic delivery of LPS that was indeed responsible for caspase-11 activation. In the other study, Hagar *et al.* found that lysates of Gram-negative but not Gram-positive bacteria, when transfected into mouse macrophages, could activate the caspase-11 pathway. The activity was also nailed down to LPS. Both studies demonstrated that cytosolic delivery of LPS through transfection or *Listeria monocytogenes* infection was sufficient to induce caspase-11 processing and the resulting cell pyroptosis.

LPS contains three structural moieties, lipid A, a core oligosaccharide chain and a variable polysaccharide chain (O antigen) [19] (Figure 1). The most conserved lipid A is responsible for caspase-11 ‘non-canonical inflammasome’ activation. Similar to LPS activation of TLR4 [20,21], tetra-acylated lipid IVa appeared to be a poor agonist; consistently, lipid IVa-producing bacteria, such as *Francisella novicida*, *Yersinia pestis* (grown at 37 °C) or an engineered *E. coli* mutant, did not trigger pyroptosis [17**,18**].

Figure 1



Innate immune recognition of cytosolic LPS by inflammatory caspases. Intracellular Gram-negative bacteria can release LPS directly into the cytosol, and this requires bacterial escape from the vacuole or vacuole lysis by IFN-induced GTPases. Circulating LPS from extracellular bacteria or direct injection in the endotoxic shock model accesses the cytosol likely through an unknown route of endocytosis. Cytosolic LPS is detected by inflammatory caspases including caspase-4/caspase-5 in human and caspase-11 in mouse, through a direct and specific binding between lipid A in LPS and the CARD in the caspase. LPS binding stimulates oligomerization and consequently proximity-induced activation of caspase-4/caspase-5/caspase-11. Activated caspase-4/caspase-5/caspase-11 then triggers cell pyroptosis and also canonical NLRP3/ASC inflammasome activation for IL-1β/18 release, both of which are critical for anti-bacteria defense. Excessive LPS leads to hyperactivation of the caspase-4/caspase-5/caspase-11 pathway and uncontrolled inflammation, thereby determining septic shock in mice.

Direct recognition of LPS by caspase-11 and human caspase-4/caspase-5

Caspase-11 activation does not require the ASC adaptor [11]. A prevailing hypothesis then, according to knowledge learned from cell death and inflammation researches, was that an unknown CARD-containing protein serves as an intracellular LPS sensor and activates caspase-11 through CARD–CARD interaction. However, extensive efforts made in several research groups (including ours) to search for such hypothetical LPS sensor turned out to be fruitless [22^{••}]. Meanwhile, we succeeded in reconstituting bacteria-induced caspase-11 processing in macrophage extracts, which also identified LPS as the agonist (unpublished). When trying to fractionate the extracts to purify the LPS sensor, we noted that recombinant caspase-11 purified from *E. coli* was a multimer of ~600 kDa while that from insect cells was monomeric [22^{••}]. This led to a surprising finding that LPS could directly bind to caspase-11 (Figure 1) and the affinity was comparable to, if not higher than, that to the TLR4/MD2 complex [23]. The binding was highly specific; LPS showed no interaction with caspase-1/caspase-9 and other bacterial molecules like muramyl dipeptide (MDP) could not form complexes with caspase-11. Direct LPS–caspase-11 association was also observed in pyroptotic cells following LPS electroporation [22^{••}].

Human caspase-4 and caspase-5 are most homologous to caspase-11. Human monocytes express high-level caspase-4 that can also mediate cytoplasmic LPS-induced pyroptosis [22^{••}]. In fact, caspase-4 and caspase-11 could functionally substitute each other in the corresponding mouse or human cells. Recombinant caspase-4 purified from *E. coli* and insect cells also exhibited differential oligomerization states, echoing that observed with caspase-11. Caspase-4 as well as caspase-5 could bind to LPS in a manner indistinguishable from caspase-11.

Consistent with the full function of lipid A in triggering pyroptosis, lipid A alone is sufficient to bind to caspase-4/caspase-11. Notably, the binding is mediated by the CARD in caspase-4/caspase-11 (Figure 1), which differs from other CARDS that generally perceive upstream signals through CARD–CARD interactions [24]. The exact mechanism for caspase-4/caspase-11 recognition of LPS is unknown in the absence of structural data. The fatty acid chain in lipid A shall play a critical role in this process, which is also the case in LPS binding to the TLR4/MD2 complex [21].

LPS binding induces caspase-4/caspase-11 oligomerization and activation

Consistent with the oligomeric state of *E. coli*-purified caspase-4/caspase-11, LPS binding of monomeric caspase-4/caspase-11 or even the CARDS alone induced their oligomerization [22^{••}] (Figure 1). As a result, caspase-4/caspase-11 became catalytic active in hydrolyzing

the zVAD-AMC substrate. Mutations in several basic residues in the CARD of caspase-11, particularly Lys-19, which disrupted LPS binding, also inhibited LPS-induced caspase-11 oligomerization, activation as well as stimulation of macrophage pyroptosis. This suggests that binding to LPS is essential for caspase-11 functioning. Lipid IVa and penta-acylated LPS from *Rhodobacter sphaeroides* (LPS-RS), though binding to caspase-11 well, could not induce its oligomerization and therefore resisted cytosolic immune detection by the caspase [17^{••},18^{••},22^{••}]. This explains the immune escape seen with certain bacteria such as *F. novicida* and *Y. pestis*, and also supports that LPS binding-induced oligomerization is required for caspase-4/caspase-11 activation (Figure 1).

The stoichiometry of LPS and caspase-4/caspase-11 binding and how the binding induces caspase activation are unknown. LPS binding may drive conformational changes of the caspases that then undergo oligomerization. Alternatively, the aggregation-prone property of LPS may bring the caspases together. In both situations, multiple caspase molecules are brought into close proximity, rendering a catalytically active conformation. Such mode of activation has been proposed for other caspases including caspase-1/caspase-9 [6,25]. Different from caspase-1/caspase-9 that are autoprocessed into the p20 and p10 fragments, caspase-11 activation is often accompanied by appearance of a p30 fragment of an undefined nature. There are also strong evidences suggesting that caspase-4/caspase-11 are activated in the absence of autoprocessing [17^{••},22^{••}].

Regulation of inflammatory caspases sensing of LPS

LPS robustly stimulates caspase-11 transcription through the TLR4 signaling [17^{••},18^{••},26]. Other TLR agonists, such as polyinosinic–polycytidylic acid, and interferon (IFN)- β/γ could also induce caspase-11 expression [13,14,15[•],16,27]. Given the low basal expression of caspase-11, transcriptional induction is important for macrophage sensing of intracellular LPS. Supporting this notion, the TRIF–IRF–IFN signaling was shown to be essential for caspase-11 activation by bacterial infections [13,14,27]. However, chemical transfection or electroporation of LPS directly into the cytosol was sufficient to robustly activate the caspase-11 pathway [17^{••},18^{••},22^{••}]. The inconsistency has been reconciled by identification of IFN-inducible GTPases (GBPs) as critical factors in the caspase-11 pathway [28[•],29[•]]. The TRIF–IRF signaling induces the expression of GBPs through type I IFNs; GBPs acts at least partially through promoting lysis of the bacteria-containing vacuole. It is possible that there might be other mechanisms regulating caspase-11-mediated innate immunity.

Caspase-11 is mainly expressed in macrophages despite a recent report of LPS sensing in mouse intestinal epithelia

[30[•]]. The situation with caspase-4/caspase-5 in human is different. The physiological function of caspase-5 is currently unknown. Caspase-4 is expressed in diverse cell types including monocytes, keratinocytes, epithelial cells and promyelocytic leukemia cells, which all showed robust responses to cytoplasmic LPS [22^{••}]. Further different from caspase-11, caspase-4 was constitutively expressed to a high level and not subjected to transcriptional induction by LPS and IFN [31]. Interestingly, the caspase-4 signaling is targeted by bacteria that have been adapted to the cytosol environment; *Shigella flexneri* encodes a type III secretion effector OspC3 that could directly bind to caspase-4 and block its activation [32].

Function of intracellular LPS sensing and LPS-induced sepsis

The molecular mechanism underlying caspase-4/caspase-5/caspase-11 activation-induced pyroptosis is unknown. The similar morphology between caspase-4/caspase-5/caspase-11 and caspase-1-mediated pyroptosis indicates a possible common death program involving proteolytic cleavage of certain cellular proteins. In host defense, caspase-1-mediated pyroptosis serves as an innate immune effector mechanism in clearing intracellular infection [33], and this probably also applies to LPS-induced caspase-11/caspase-4 activation (Figure 1). Supporting this idea, *Caspase-11* deficiency rendered the mice sensitive to lethal challenge with *B. thailandensis* and *B. pseudomallei* [15[•]]. Caspase-11 and caspase-4 are also functional in non-macrophage cells; their activation could limit *S. typhimurium* replication in cultured intestinal epithelial cells and promote shedding of infected epithelia to control bacterial growth in mice [30[•]]. In *L. pneumophila* infection, it was proposed that caspase-4/caspase-5/caspase-11 activation could promote fusion of the bacteria-containing vacuole with the lysosome [12]. Several studies have established that caspase-11 activation signals the canonical NLRP3 inflammasome activation and induce IL-1 β secretion [11,13,14,17^{••},18^{••},27,28[•],30[•]]. Despite an unknown mechanism, activation of the NLRP3 inflammasome by caspase-11 shall also contribute to anti-bacterial defenses. Notably, many caspase-4-expressing cells do not possess the NLRP3 inflammasome [22^{••}]. Thus, indirect activation of IL-1 β secretion is not a general function of caspase-4/caspase-5-mediated innate immunity. Notwithstanding, it is possible that caspase-4/caspase-5/caspase-11 may regulate other cytokines or inflammatory factors such as IL- α in certain cell types.

Excessive LPS leads to endotoxic shock in mice, an established model for Gram-negative bacteria-induced sepsis. Early studies showed that loss of either *caspase-1* or *caspase-11* in mice rendered resistance to LPS-induced death [34,35]. Clarification of *caspase-11* polymorphism in different mouse strains suggests that caspase-11 rather than caspase-1 dominates the LPS lethality [11]. *Tlr4*-deficient mice are also known to resist endotoxic

shock. Hagar *et al.* and Kayagaki *et al.* have now demonstrated that priming of the mice with TLR3 ligand to upregulate caspase-11 expression can bypass the requirement of TLR4 [17^{••},18^{••}]. Thus, the role of TLR4 in mouse endotoxic shock is largely limited to stimulate caspase-11 expression. Moreover, caspase-4/caspase-5 have different expression profiles from that of caspase-11. Such difference, together with the re-defined role of TLR4, provides plausible explanations for the failure of using TLR4 antagonist Eritoran to treat sepsis. Despite the importance of caspase-11 in endotoxic shock, two other important questions remain. How does excessive LPS-induced caspase-11 activation lead to organ failure and death of the mice and what is the role of pyroptosis in this process? Secondly, how does LPS get into the cytosol of target cells in the absence of TLR4-mediated endocytosis [17^{••},18^{••},36]? These shall all be important area of future investigation.

Conclusions and perspectives

A 'non-canonical inflammasome' for caspase-11 activation was originally proposed based on the high similarity between caspase-11 and caspase-1 [11]. Recent studies have not only identified cytoplasmic LPS being the agonist for the 'non-canonical inflammasome' [17^{••},18^{••}], but also revealed that inflammatory caspases (caspase-4/caspase-5/caspase-11) themselves are direct sensors for LPS [22^{••}]. Cytosolic recognition of LPS induces caspase oligomerization and activation, thereby triggering cell pyroptosis for mounting anti-bacterial defenses (Figure 1). Identification of caspase-4/caspase-5/caspase-11 being LPS receptors is conceptually unexpected, highlighting a high diversity in cytosolic innate immune detection. The mode of caspase-4/caspase-5/caspase-11 action to some extent is similar to factors C and G, two serine proteases in horseshoe crab that are activated by LPS and β -glucan for initiating anti-bacterial defense [37], which together defines a pattern recognition receptor protease family. Discovering intracellular LPS receptors brings a new angle for future studies on bacterial infection and host immunity. The critical role of caspase-11 in endotoxic shock also opens a new avenue for development of anti-sepsis therapeutics. Beside these, the structural mechanism and functional regulation of LPS activation of the inflammatory caspases as well as the molecular/physiological programs leading to pyroptosis and septic shock shall also be the subjects of future investigations.

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